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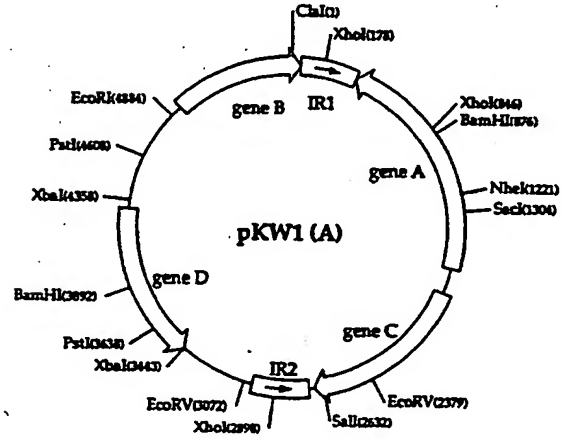

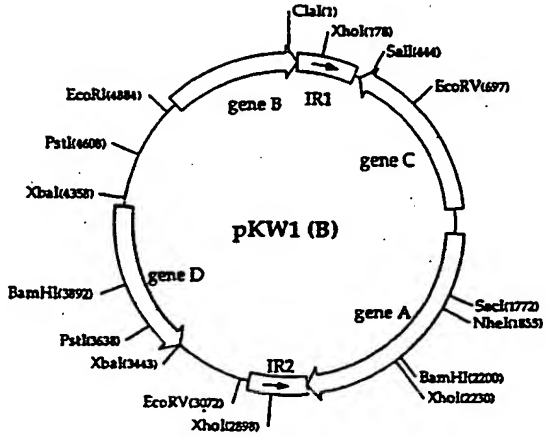

(57) Abstract

The invention discloses a new yeast plasmid, new cloning and/or expression vectors derived from said plasmid, and use thereof.

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The present invention concerns a new yeast plasmid, as well as new cloning and/or expression vectors derived therefrom, their preparation and their use, notably for the production of recombinant proteins. The invention also concerns recombinant host cells containing such vectors.

More specifically, the present invention concerns all or part of a new plasmid isolated from the yeast *Kluyveromyces waltii* or a derivative thereof, and the cloning and/or expression vectors constructed from it.

During the past ten years, yeasts have become very promising host microorganisms for the production of heterologous proteins.

In particular, the identification by Beggs et al. (Nature 275 (1978) 104) of the plasmid 2μ and vectors derived therefrom was one of the keys of the current development of the genetic and molecular study of the yeast *Saccharomyces cerevisiae*. Since that time, the 2μ system has allowed the introduction and the expression of heterologous genes in yeast to obtain proteins of pharmaceutical or agro food interest. However, plasmid 2μ and its derivatives can replicate effectively only in yeasts belonging to the species *S. cerevisiae* and to some related species. Therefore, this system cannot be used for genetic manipulation in most yeast species. Notably, it cannot be used in yeasts whose physiological properties, which are different from those of *S. cerevisiae*, would allow better-performing new applications and/or industrial uses.

Different laboratories therefore have sought other plasmids in different yeast species. Thus, several circular plasmids have been found in the yeasts of the genus *Zygosaccharomyces*, and notably the plasmids pSR1 and pSR2 (Toh-é et al., J. Bacteriol. 151 (1982) 1380); pSB1, pSB2, pSB3 and pSB4 (Toh-é et al., J. Gen. Microbiol. 130 (1984) 2527); and pSM1 (Utatsu et al., J. Bacteriol. 169 (1987) 5537). A circular plasmid has also been found in *Kluyveromyces drosophilarum*: pKD1 (Falcone et al., Plasmid 15 (1986) 248).

All these plasmids present characteristics which they share with plasmid 2μ, notably inverted repeat sequences, and the existence of two possible isomeric forms due to a site-specific recombination site.

However, these plasmids always present the drawback of having a narrow host spectrum. Thus, because of their specificity with respect to yeast hosts, these plasmids can only be used in a limited number of strains.

The present invention is the result of the identification of a natural plasmid of the yeast *Kluyveromyces waltii*. This plasmid, called pKW1, is the first known natural plasmid of this yeast species. The plasmid has been purified from the strain *K. waltii* CBS 6430, and it has been mapped using restriction enzymes; the resulting map is presented in Figure 1.

An object of the invention thus consists of the plasmid pKW1 isolated from the strain *K. waltii* CBS 6430, or any fragment or derivative thereof.

In the meaning of the invention, the term derivative denotes plasmids which, in spite of some modifications, preserve the properties of the original plasmid. Notably, the modifications can take on the form of mutations or deletions concerning regions having a relatively large size. They can also be insertions or suppressions, for example, of cloning sites.

The term pKW1 fragment notably denotes the different genetic elements of this plasmid. It is more advantageous to notably mention, as genetic elements of pKW1, the structural genes or parts thereof, functional promoter sequences, inverted repeat sequences, or sequences (IR) that allow replication (replication origin) or confer stability to the plasmid (stability locus).

Indeed, the structural study of plasmid pKW1 has allowed the detection of analogies with the plasmid 2μ of *S. cerevisiae*. Thus, 4 structural genes have been identified (see Figure 2), as well as a replication origin. Moreover, the cloning of pKW1 in *E. coli* has allowed the isolation of 4 types of recombinant plasmids, corresponding to 2 isomeric forms of plasmid pKW1 (forms A and B) cloned in the vector pKan21 in the two possible orientations (vector pKan21 is described in Example 3.1). These two forms are presented in Figure 1. The existence of two isomeric forms indicates the presence of inverted repeat sequences. The study has thus shown that plasmid pKW1 contains a pair of inverted repeat sequences each having a length of 0.3 kb, and two unique sequences of 2.5 and 2.3 kb, whose orientation distinguishes the two isomeric forms A and B. The molecular size of plasmid pKW1 is thus approximately 5.5 kb. Different restriction sites have been detected, for example, the following sites: EcoRI, SphI, SalI, ClaI, NheI and BglI.

The complete nucleotide sequence of plasmid pKW1 has also been determined (Figure 3). The absence of homology between this sequence and that of known plasmids has been demonstrated, notably by hybridization experiments (see Example 2). The absence of hybridization under conditions of moderate stringency is characteristic of this difference in sequence.

In a preferred embodiment, the invention relates to a plasmid comprising all or part of the sequence presented in Figure 3 or a derivative thereof.

Moreover, the applicant has also shown that it is possible to use plasmid pKW1 or fragments thereof to construct particularly stable cloning and/or expression vectors.

Another object of the present invention thus consists of cloning and/or expression vectors characterized in that they comprise all or part of plasmid pKW1 of *K. waltii* CBS 6430 represented in Figure 1, or a derivative thereof.

A more specific object of the invention consists of a cloning and/or expression vector characterized in that it comprises at least one genetic element of plasmid pKW1.

Because of the host spectrum of plasmid pKW1, the vectors of the invention can be used in species other than the natural host *K. waltii*.

They can notably be used for the transformation of a large variety of species, notably yeast species.

Different types of vectors have been constructed from pKW1; they differ at the level of the size of the fragment originating from pKW1 and thus of the functional elements originating from pKW1.

An even more specific object of the invention consists of a cloning and/or expression vector characterized in that it comprises the replication origin of plasmid pKW1.

Other constructions can be prepared, containing fragments of varying sizes, to study the influence of the different elements of pKW1 on the stability of the vectors, their host specificity, and their effectiveness for the expression of the heterologous genes. In particular, expression vectors can be elaborated from the different genetic elements of plasmid pKW1 (replication origin, inverted repeat sequences, structural genes, promoter regions...), which one can introduce into known plasmids to improve their performances or to confer novel properties to them. In the same manner, vectors can be obtained by adding elements to plasmid pKW1, or by replacing certain genetic elements of pKW1 with elements originating from other plasmids. Thus, vectors can be obtained by substituting, for example, the replication origin of pKW1 with the replication origin of the plasmid 2μ of *S. cerevisiae* or of the plasmid pKD1 of *Kluyveromyces*, or by a chromosomal replicon (ARS) of yeast (example: KARS of *K. lactis*). In the same manner, vectors can be obtained by substituting the stability locus of pKW1 with that of plasmid 2μ of *S. cerevisiae* or plasmid pKD1 of *Kluyveromyces*. It can be particularly advantageous to prepare hybrid vectors which comprise plasmids pKD1 and pKW1.

Advantageously, the vectors of the invention comprise the entire plasmid pKW1 as represented in Figure 1.

It is preferred for the vectors according to the invention to comprise plasmid pKW1, linearized at a functionally neutral restriction site.

The term functionally neutral restriction site, in the meaning of the present invention, denotes a restriction site at whose level it is possible to interrupt the structure of the plasmid without altering its replication and stability properties.

In particular, the sites can be sites which are present on plasmid pKW1. As an example, one can notably mention the sites ClaI(1); PstI(4608); or EcoRV(3072) as represented in Figure 1.

The sites can also have been artificially introduced into plasmid pKW1, or rendered unique. In this case, the sites are preferably introduced in intergenic regions of the plasmid, and notably in the region located between the genes B and D, or in the region located between gene D and IR2.

Advantageously, according to the present invention, plasmid pKW1 is linearized at the level of a unique restriction site.

A particularly interesting site in this regard is the unique ClaI site located in position 1 in Figure 1. Indeed, the applicant has shown that this site allowed the use of plasmid pKW1 to construct the cloning and/or expression vectors, by introducing at this level, for example, heterologous DNA fragments, while maintaining the stable replication of the vector obtained. This result is surprising to the extent that site ClaI is located in structural gene B.

The use of such neutral cloning sites thus allows the obtention of very stable vectors which are capable of staying in the transformed cells, even in the absence of any selective pressure.

Advantageously, the vectors of the invention contain, in addition, a heterologous DNA sequence comprising at least one structural gene, under the control of signals allowing its expression.

The signals allowing the expression of the structural gene(s) can consist of one or more elements chosen from promoters, terminators or secretion signals. It is understood that the signals are chosen as a function of the used host, the structural gene and the desired result. In particular, it can be preferable in some cases to use a promoter which can be regulated, allowing the decoupling between the growth phases of the hosts and the phase of expression of said structural gene(s). Similarly, the use of the peptide signal (secretion signal) can make it possible to increase the production rate of the desired protein and facilitate the purification step.

It is preferred for the promoters used to be derivatives of yeast genes. Promoters of particular interests are those derived from glycolytic genes of yeasts of the genus *Saccharomyces* or *Kluyveromyces*. Notably, one can cite the promoters of genes coding for phosphoglycerate kinase of *S. cerevisiae* (PGK), glyceraldehyde-3-phosphate dehydrogenase (GPD), enolases (ENO), alcohol dehydrogenases (ADH). One can also cite promoters derived from strongly expressed genes, such as the lactase gene (LAC4) or the acid phosphatase gene (PHO5).

Moreover, these genes can be modified by mutagenesis, for example, to add additional transcription control elements, such as, notably, UAS ("Upstream Activating Sequence") regions.

The structural gene which can be introduced into the vectors of the invention preferably codes for a polypeptide of pharmaceutical or agro food interest. For example, one can mention enzymes, such as, notably, superoxide dismutase, catalase, amylases, lipases, amidases, chymosine, etc.), blood derivatives (such as serum albumin, alpha- or beta-globin, factor VIII, factor IX, van Willebrand factor, fibronectin, alpha-1 antitrypsin, etc.), insulin and its variants, lymphokines (such as interleukines, interferons, colony stimulation factors (G-CSF, GM-CSF, M-CSF...), TNF, TRF, etc.), growth factors (such as growth hormone, erythropoietin, FGF,

EGF, PDGF, TGF, etc.), apolipoproteins, or antigenic polypeptides for the manufacture of vaccines (hepatitis, cytomegalovirus, Epstein-Barr, herpes, etc.).

In a special embodiment of the invention, the structural gene can be a gene resulting from the fusion of several DNA sequences. The gene can notably be a gene coding for a hybrid polypeptide containing an active part associated with the stabilizing part. As an example, one can mention the fusion between albumin or albumin fragments and a receptor part of a virus receptor (CD4, etc.).

In another embodiment, the heterologous DNA sequence can comprise several structural genes, notably genes involved, at the genetic or biochemical level, in the biosynthesis of a metabolite. The metabolite can be, in particular, an antibiotic, an amino acid or a vitamin.

In a special embodiment, the vectors of the invention contain, in addition:

- an *E. coli* replicon and/or
- at least one selection marker.

These elements allow the manipulation of the vectors of the invention in a much easier manner.

Another object of the invention concerns recombinant cells containing a vector as defined above.

The recombinant cells are preferably chosen from yeasts.

The applicant has shown that the vectors of the invention can indeed be used either in *K. waltii* (the natural host of pKW1) or in yeasts of different species or even different genera. Notably, they can be used in other *Kluyveromyces* species or in *Saccharomyces*. Moreover, when strain *K. waltii* CBS 6430 is used as the host cell, homologous recombinations between the vectors of the invention and the resident plasmid pKW1 can affect the stability of the vectors, and thus decrease the performances of the host/vector pair. To further improve the stability of the vectors of the invention in such a host/vector pair, the applicant has prepared a strain *K. waltii* pKW1$^-$ (KW18). This strain allows the optimization of the industrial use of the vectors of the invention (see Example 4).

Different techniques can be used to introduce the vectors of the invention into the host cells. In particular, transformation (Bianchi et al., Curr. Genet. 12 (1978) 185) and electroporation (Delorme, Appl. Environ. Microbiol. 155 (1989) 2242) yield good results. However, it is clear that the invention is not limited to a particular technique.

An object of the invention also consists of a method for the preparation of a polypeptide according to which a recombinant cell as defined above can be cultured and the produced polypeptide recovered. More specifically, the method of the invention allows the production of proteins of pharmaceutical or agro food interest, such as those indicated above. More

specifically, the method of the invention can be adapted to the production of human albumin and of its variants or precursors.

In the case where the structural genes are involved in the biosynthesis of a metabolite, the recombinant cells can also be used directly in a bioconversion process.

Other advantages of the invention will become apparent after a reading of the following examples which must be considered to be illustrative and nonlimiting.

Legend of the figures

Figure 1: Restriction map of plasmid pKW1. The inverted repeat sequences as well as the structural genes A-D are indicated. The positions indicated for the restriction sites correspond to the first nucleotide recognized by the enzyme.

Figure 2: Study of the open frames of plasmid pKW1. The genetic elements indicated are localized at the following positions with reference to the sequence presented in Figure 3: Gene A: nucleotides 1454-2755; gene B: nucleotides 4948-54; gene C: nucleotides 389-1309 on the complementary strand; gene D: nucleotides 3444-4313 on the complementary strand; IR1: nucleotides 53-368; IR2: nucleotides 2713-3028.

Figure 3: Nucleotide sequence of plasmid pKW1. The sequence represented corresponds to the B form of the plasmid. Position 1 corresponds to the first nucleotide of the sequence recognized by the enzyme ClaI. Plasmid pKW1 was fragmented by restriction enzymes, and the fragments were cloned in pTZ18R (Pharmacia). The sequences of the cloned segments were determined using the 2-strand Sanger method.

Figure 4: Restriction maps of the shuttle plasmids YIP5 and pKan21. Ap: ampicillin resistance gene; Tc: tetracycline resistance gene; Km: kanamycin resistance gene (G418); LacZ: structural gene of β-galactosidase.

Figure 5: Strategy of construction of the vectors pBNA1, pNEA2, pBNB1/A3, pNEB1, pXXY2 and pXXK3. See also Table 1.

Figure 6: Restriction map of the vector pXXK3.

Figure 7: Strategy of construction of the vectors pKWC11, pKWS1 and pKWS14.

Figure 8: Study of the stability of vectors pKWC11 and pXXK3 in the strain *K. waltii* KW18. In each case, a transformed clone was cultured in nonselective YPG medium for the indicated number of generations, and then aliquots of the culture were plated on YPG agar Petri dishes with and without G418, to determine the total number of cells and the number of cells resistant to G418. The stability corresponds to the % of resistant cells.

Figure 9: Strategy of construction of the expression vector pXPHO5. Abbreviations: P = promoter, T = terminator, ss = secretion signal, CIP = Calf Intestinal Phosphatase, Km = kanamycin, E = EcoRI, H = HindIII, S = SalI, B = BamHI, Sm = SmaI.

Figure 10: Restriction maps of the vectors pXKN18 and pXPHO5. Legend: see Figure 9.

Figure 11: Immunological detection of IL1β produced by *K. waltii*. The molecular weight markers (KDa) are indicated on the left. Well 1: Reference IL-1β (100 ng); well 2: culture supernatant of the transformant pXKN18 (without IL-1β cassette); well 3: culture supernatant of the transformant pXPHO5 treated with endo-N-acetylglucosamidase H; wells 4 and 5: supernatants of the cultures of the transformants pXPHO5 in LPi and HPi medium, respectively.

Figure 12: Strategy of construction of the plasmid pYG65.

Figure 13: Strategy of construction of the plasmid pYG70.

Figure 14: Strategy of construction of the plasmid pYG141. aph: gene coding for aminoglycoside 3'-phosphotransferase, which confers resistance to kanamycin; bla: gene coding for the β-lactamase which confers resistance to ampicillin.

Figure 15: Strategy of construction of the plasmid pYG142.

Tables 1 and 2: Composition of vectors derived from pKW1 according to the invention. (*) Cloning sites.

Tables 3, 4 and 5: Transformation by the vectors of the invention of *S. cerevisiae*, *K. waltii* and different strains of *Kluyveromyces*, respectively. The stability of the transformants is expressed by the percentages of Ura+ cells after 10 generations of growth in nonselective YPD medium. The isonuclear strains *K. waltii* $pKW1^+$ and $pKW1^-$ are CBS 6430 and KW18, respectively.

General cloning technique

The classical methods of molecular biology, such as centrifugation of plasmid DNA in a cesium chloride-ethidium bromide gradient, digestion by restriction enzymes, gel electrophoresis, electroelution of DNA fragments from agar gels, transformation in *E. coli*, etc., are described in the literature (Maniatis et al., "Molecular Cloning: a Laboratory Manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1986; Ausubel et al. (eds.), "Current Protocols in Molecular Biology," John Wiley & Sons, New York 1987).

In vitro mutagenesis directed by oligodeoxynucleotides is carried out according to the method developed by Taylor et al. (Nucleic Acids Res. 13 (1985) 8749-8764) using the kit distributed by Amersham. Sequencing of nucleotides is carried out according to the dideoxy technique described by Sanger et al. (Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467). Enzymatic amplification of specific DNA fragments is carried out by the PCR ("Polymerase-catalyzed Chain Reaction") reaction under the conditions described by Mullis and Faloona (Meth. Enzym., 155 (1987) 335-350) and Saiki et al. (Science 230 (1985) 1350-1354), using a "DNA thermal cycler" (Perkin-Elmer Cetus) following the recommendations of the manufacturer.

Examples

1) Isolation and purification of pKW1

Strain CBS 6430 is cultured in 2 L of YPG medium (yeast extract 1%, Bactopeptone 1%, glucose 2%) with stirring at 26°C for approximately 18 h. The cells in the early stationary phase are harvested by centrifugation. 13-15 g of cell mass per liter are usually obtained. The cells are washed with 150 mL of 1M sorbitol containing 30 mg of zymolyase 20T (Kirin Breweries Co., Tokyo). After incubation at 30°C for 1 h, one adds to the suspension of protoplasts 5 mL of 10% sodium dodecyl sulfate and 5 mL of 0.5M EDTA, pH 7.0. The mixture is immediately vigorously stirred and incubated at 50°C for 1-2 h. Potassium acetate is added to the lysate to obtain a final concentration of 1M and the mixture is maintained in ice for 2 h. The precipitates formed are eliminated by centrifugation (Sorvall SS34, 15,000 rpm, 30 min). The supernatant, to which 2 volumes of 95% ethanol are added, is cooled in ice to precipitate the nucleic acids. The precipitates are collected by centrifugation, washed with ethanol at 70%, dried under a vacuum, and finely dissolved in 40 mL of 5 x TE (1x TE is 10mM tris-HCl, 1mM EDTA, pH 8). After the addition of 40 g of CsCl in 5 mL of ethidium bromide (stock solution of 10 mg/mL), the mixture is centrifuged at 60,000 rpm for 6 h (Beckman, rotor 60 Ti). The fluorescent band of the plasmid DNA is located below the major band of the chromosomal DNA. The DNA of the plasmid is collected and subjected to the second centrifugation cycle in CsCl/ethidium bromide. The collected plasmid DNA is mixed with one volume of isopropanol which has first been equilibrated with 4M CsCl to eliminate the ethidium bromide. After several extractions with isopropanol, the DNA solution is dialyzed against 1 x TE. The volume of the solution can be reduced by dialysis against polyethylene glycol 6000 in flakes.

2) Sequencing and studies of homologies

Plasmid pKW1 was sequenced using the method described by Sanger et al. (Proc. Natl. Acad. Sci. USA 74 (1977) 5463-5467). The complete sequence is indicated in Figure 3.

The absence of a homology between this sequence and that of known plasmids was determined by molecular hybridization experiments: pKW1 is labeled with ^{32}P and hybridized with the following plasmids, which were first immobilized on a nitrocellulose filter:

- plasmid 2μ from *S. cerevisiae*,
- pSR1, pSB3 and pSB4 from *Z. rouxii*,
- pSB1 and pSB2 from *Z. bailii*, and
- pKD1 from *K. drosophilarum*.

The hybridization was carried out under moderately stringent conditions (0.6M Na^+, 65°C, 18 h); none of the plasmids produced a positive signal of hybridization.

The other circular plasmids (pSB1, pSB4) whose sequences have not yet been described are also different from pKW1, in the molecular size, the length of the inverted repeats, and in their host species.

3) Constructions of cloning vectors derived from pKW1

Two types of recombinant molecules were constructed from pKW1.

3.1 In the first type, different fragments of pKW1 (corresponding, for example, to the genetic elements of pKW1) were introduced into shuttle vectors, and notably into the vectors YIp5 (Struhl et al., Proc. Nat. Acad. Sci USA 76 (1979) 1035) and pKan21, represented in Figure 4.

Plasmid pKan21 was constructed by insertion of the gene aph (Genblock, Pharmacia) which confers resistance to kanamycin (G418) in the form of a 1.25 kbp AccI fragment, in the NarI site of plasmid pUC19 (Viera and Messing, Gene 19 (1982) 259). Thus, pKan21 contains, in addition to aph, the gene bla which confers resistance to ampicillin and the replication origin of ColE1 which allows replication in *E. coli*. The yeasts transformed by the vectors derived from pKan21 can be detected by their growth on a medium containing 200 µg/mL of geneticine (G418).

Plasmid YIp5 is a derivative of plasmid pBR322 in which the gene URA3 from *S. cerevisiae* was inserted as a selection marker. The yeast, transformed by the derivatives of YIp5, is detected by its growth on a uracil-free medium. In the last case, the host yeast is an auxotroph which is deficient in orotidine-monophosphate carboxylase of the uracil synthesis pathway.

The vectors of this first type are pBNA1, pNEA2, pBNB1/A3, pNEB1, pXXY2 and pXXK3 (Table 1 and Figure 4).

- Vector pBNA1

pKW1 is digested with BglII and NheI (see Figure 5) and the DNA fragments are separated by electrophoresis. The 2.4-kbp fragment is recovered and inserted by ligation between the unique BamHI and NheI sites in the tetracycline resistance gene of plasmid YIp5 (Yanish-Peron et al., Gene 33, 1985, 103-119), where the latter was first digested by BamHI and NheI and repurified. The BglII and BamHI ends are compatible for a specific ligation. *E. coli*, transformed by the ligation mixture, is selected on LB agar medium which contains ampicillin. The insertion is verified by replication of the transformants on LB agar medium containing tetracycline, as the latter are sensitive to this antibiotic. The structure of the plasmid is verified by extraction of the DNA from individual transformants and its analysis using restriction enzymes. For example, plasmid pBNA1, digested by PstI, produces 3 fragments of 3.28, 3.15 and 1.36 kbp: double digestion with PstI/NheI yields 4 fragments of 3.15, 2.3, 1.36 and 1.0 kbp.

- Vector pNEA2

The 2.0 kbp NheI-EcoRI fragment of pKW1 (see Figure 5) is isolated and inserted between the two unique NheI and EcoRI sites in the tetracycline resistance gene of YIp5, where the latter was first digested by these two enzymes. The ligation product is introduced into *E. coli* and the transformants which are resistant to ampicillin and sensitive to tetracycline are isolated. The plasmid is isolated, as in the case of pBNA1, from one of its transformants. The structure of the recombinant plasmid obtained is verified by restriction. For example, digestion by PstI produces 2 fragments of 4.35 and 3.15 kbp; double digestion with PstI/XhoI produces 4 fragments of 3.15, 1.8, 1.75 and 0.7 kbp.

- Vector pXXY2

The 545 bp XhoI-XbaI fragment of pKW1 (see Figure 5) is isolated and inserted between the unique SalI-NheI sites in the tetracycline resistance gene of plasmid YIp5. The XhoI and SalI ends, on the one hand, and XbaI and NheI ends, on the other hand, are compatible for a specific ligation. The ligation product is introduced into *E. coli*, and the recombinant plasmid is isolated as in the case of pBNA1. The structure of the plasmid obtained is verified by restriction. For example, digestion with EcoRI + NruI produces two fragments of 4.5 and 0.96 kbp.

- Vector pXXK3

The 545 bp XhoI-XbaI fragment of pKW1 (see Figure 5) is isolated and inserted between the unique SalI-XbaI sites (polylinker at the N terminal of LacZ) of plasmid pKan21. The ligation mixture is introduced into *E. coli*, and the transformants, which are plated on LB medium containing X-gal and IPTG, are isolated as white colonies among blue colonies. When replicated on LB medium containing kanamycin, they grow well. These colonies are individually analyzed for their plasmid content. The plasmid obtained from one of the transformants presents the structure shown in Figure 6, which was verified by restriction. For example, digestion with BamHI + PstI produces four fragments: 2.5, 1.3, 0.55 and 0.19 kbp.

- Vector pBNB1/A3

The 1.9 kbp BglII-NheI fragment of pKW1 (see Figure 5) is isolated and inserted between the BamHI-NheI sites of YIp5. The recombinant plasmid is isolated as in the case of pBNA1. The structure of the plasmid is verified by restriction. For example, digestion with PstI produces 3 fragments of 3.15, 2.8 and 1.36 kbp; double digestion with PstI/NheI produces 4 fragments of 3.15, 1.8, 1.36 and 1.0 kbp.

- Vector pNEB1

The 2.5 kbp NheI-EcoRI fragment of pKW1 (see Figure 5) is isolated and inserted between the NheI-EcoRI sites of YIp5. The recombinant plasmid is isolated as in the case of pBNA1. The structure of the plasmid is verified by restriction. For example, digestion with PstI produces 2 fragments of 4.65 and 3.15 kbp; double digestion with PstI/XhoI produces 3 fragments of 3.15, 2.9 and 1.75 kbp.

3.2 The second type of recombinant molecules contains the totality of the sequence of pKW1. To obtain these vectors, pKW1 is linearized by a unique cut at a restriction site, allowing the introduction of heterologous DNA segments. Such segments can contain structural genes, included, for example, in expression cassettes and/or whole shuttle vectors, such as, notably, pKan21 or YIp5 (Figure 4).

Examples of this type of vectors are pKWC11, pKWS1 and pKWS14 (Table 2 and Figure 7).

- Vector pKWS14

The DNA of pKW1 is digested by the restriction enzyme SalI. Plasmid pKan21, described above, is also digested by SalI (the unique SalI site is localized in the cloning multisite present in the LacZ gene). The two plasmids are ligated by DNA ligase. The ligation mixture is used for the transformation of *E. coli* JM83 as in the preceding case. The suspension of the cells transformed is plated on LB agar containing X-gal and IPTG. The white colonies, among the blue ones, are harvested individually. They are resistant to ampicillin and kanamycin. Their plasmid content is analyzed on DNA minipreparations as above. Plasmid pKWS14, isolated from one of the transformants, contains form A of plasmid pKW1 and it has the structure shown in Figure 7. It is verified by restriction. For example, digestion with BamHI produces 3 fragments of 5.6, 2.6 and 1.2 kbp.

- Vector pKWC11

Plasmid pKW1 is digested with ClaI. Plasmid pKan21 is digested with AccI. They are repurified by treatment with phenol and precipitation with ethanol. The DNA of the two plasmids are mixed in approximately equal quantities and subjected to ligation with DNA ligase for one night. The ligation product is amplified in *E. coli*. The colonies of the transformants are white on LB medium containing Xgal and IPTG. They are resistant to kanamycin and ampicillin in the medium containing one or the other of these antibiotics. Plasmid pKWC11, isolated from one of the transformants, contains form A of plasmid pKW1 and has the structure shown in Figure 7. It is verified by restriction. For example, digestion of the plasmid with BamHI produces 3 fragments of 5.6, 2.9 and 0.9 kbp.

- Vector pKWS1

Plasmid pKW1 and plasmid YIp5 are digested with SalI. The mixture is repurified and subjected to ligation. The ligation product is introduced into *E. coli*. Transformants which are resistant to ampicillin and sensitive to tetracycline are obtained. Plasmid pKWS1, isolated from one of them, contains form A of plasmid pKW1 and it has the structure shown in Figure 7. For example, digestion of the plasmid with EcoRI yields two fragments of 8.3 and 2.7 kbp.

4) Construction of a strain CBS 6430 pKW1$^-$

K. waltii CBS 6430 was first transformed by the recombinant plasmid pKWS14 (Table 2 and Figure 7). The transformation method used is essentially that described by Chen and Fukuhara (Gene 69, 181 (1988)) using protoplasts. The transformants obtained are maintained for 75 generations on YPD agar medium 1 mg/mL G418 (YPD medium: yeast extract 10 g/L; peptone 20 g/L; glucose 20 g/L). They are then transferred to a liquid YPD medium without antibiotic, and maintained for 10 generations.

Under these conditions (without selective pressure), plasmid pKWS14 is progressively lost. The colonies which are sensitive to G418 and which appear are collected and individually tested for the presence of the plasmids. The test consists of the extraction of cellular DNA, followed by electrophoresis of these DNAs on agar gel. The presence of plasmids is determined by staining with ethidium bromide. Among the colonies which have become sensitive to G418, 25% were shown to lack any plasmid. One of these colonies was kept as a strain of *K. waltii* which lacked plasmid pKW1, and it was called KW18.

5) Transformation of different yeasts

5.1 Transformation of *S. cerevisiae*

Among the different vectors described in Tables 1 and 2 and in Figures 4 and 5, some of those containing the marker URA3 were used to transform an auxotrophic strain ura3 of *S. cerevisiae* (strain S150-2B: Mat a, ura3, leu2, trp1, his3, 2μ).

The transformation method is essentially the one described by Sherman et al. (Yeast Genetics, Cold Spring Harbor, NY, 1986).

The results obtained are indicated in Table 3. They show that the vectors of the invention are capable of transforming the yeasts of the genus *Saccharomyces*.

5.2 Transformation of *K. waltii*

The transformation of *K. waltii* was carried out with vectors which carry the marker for kanamycin resistance.

The transformation method used is essentially the one described by Chen and Fukuhara (Gene 69 (1988) 181) using protoplasts. It is clear that any other technique which allows the introduction of a DNA fragment in a microorganism can be used.

The results obtained are presented in Table 4.

They show that the vectors of the invention are capable of transforming the yeast *K. waltii* with a high frequency.

Moreover, the stability study described in Figure 8 shows that vectors can be obtained from pKW1 presenting a stability of 100% after 50 generations of growth in a nonselective medium. This is perfectly illustrated by vector pKWC11. This study also shows that it is preferable, to obtain a relatively high stability, to use the vectors which comprise only the replication origin of plasmid pKW1 in host cells having a resident pKW1 plasmid.

5.3 Transformation of other yeasts

Vector pKWC11, which is highly stable and autonomous in *K. waltii*, was used to test the transformability of different yeast species, notably those belonging to the genus *Kluyveromyces*.

The results are presented in Table 5.

The presence of vector pKWC11 in the transformants was verified by electrophoresis.

All of these results show that the range of host species for the vectors of the invention can be very large, beyond the genus *Kluyveromyces*.

6) Use of the vectors of the invention for the production of heterologous proteins

6.1 Interleukin-1β:

6.1.1 Construction of an expression and secretion vector of IL-1β derived from pKW1 (Figures 9 and 10).

- Vector pXXK3 (Table 1, Figure 6) is linearized by EcoRI, and the ends are filled with the Klenow fragment of DNA polymerase I of *E. coli*. A synthetic "linker" (5'-GCGGCCGC-3') forming a restriction site which is recognized by the enzyme NotI is added by means of T4 ligase, and the vector so obtained (pXKN18) is purified after its amplification in *E. coli* (Figure 10).

- An expression cassette of IL-1β is prepared, consisting (a) of the regulated promoter PHO5 originating from *S. cerevisiae* (Bajwa et al., Nucl. Acid. Res. 12 (1984) 7721-7739), (b) the gene of human IL-1β (Jung et al., Ann. Inst. Pasteur/Microbiol. 139 (1988) 129-146) preceded (c) by a synthetic sequence corresponding to the signal sequence of the killer toxin of pGKL1 of *K. lactis* (pre region of the gene of the alpha subunit) (Stark and Boyd, EMBO J. 5, (1986) 1995-2002), and (d) by the terminator PHO5. The expression cassette was isolated from the vector pSPHO5-IL14 whose construction is described in the patent EP 361991. The cassette

was prepared as follows: At the 5' end of the gene coding for the mature part of IL-1β, the following synthetic sequence is inserted, in the form of an EcoRI fragment:

MetAsnIlePheTyrIlePheLeuPheLeuLeuSerPheValGlnGlyLysArg

5'-AATTATGAATATATTTTACATATTTTTGTTTTTGCTGTCATTCGTTCAAGGTAAAAG-3'

3'-TACTTATATAAAATGTATAAAAACAAAAACGACAGTAAGCAAGTTCCATTTTCTTAA-5'

The last codons added (Lys and Arg) form a potential restriction site which is recognized by the endopeptidase Kex1 of *K. lactis* (Tanguy-Rougeau et al.; FEBS Lett. 234 (1988) 464). This sequence was fused to the IL-1β gene by the EcoRI site, forming the following junction:

Gly Lys Arg Ile His Met Ala

5'....GGT AAA AGA ATT CAT ATG GCA ....3'

Alanine (GCA) corresponds to the first amino acid of mature IL-1β. Arg-Ile-His-Met corresponds to an EcoRI-NdeI "linker" introduced to facilitate the cloning (see EP 361 991).

The entire cassette is put in the form of a NotI fragment by the addition of a corresponding linker (5'-GCGGCCGC-3').

- The secretion cassette of IL-1β is inserted, at the NotI site, in pXKN18. The resulting vector is called pXPHO5 (Figure 10).

6.1.2 The strain *K. waltii* CBS 6430 is transformed with vector pXPHO5, under the conditions described in Example 5.2.

6.1.3 Expression of IL-1β:

The transformed cells are cultured at 28°C, in the absence of G418, in the liquid medium LPi (low mineral phosphate content) and the medium HPi (high phosphate content), prepared according to Chen and Fukuhara (Gene 69 (1988) 181-192), for 4 days. 50 mL of culture are centrifuged and the supernatants are filtered through a Millipore membrane (0.22 µm). The proteins are precipitated by the addition of ethanol at the final concentration of 60%. The precipitates are dissolved in 2 mL of Laemmli buffer (Nature 227 (1970) pp. 680-685) and 20-µL samples are used for SDS-PAGE analysis according to Laemmli (document cited above). After the electrophoresis, the proteins are transferred to a nitrocellulose sheet, and treated with a polyclonal anti-human IL-1β rabbit antiserum. The blot is then treated with a second biotinylated

anti-rabbit polyclonal antibody (Vectastain ABC ImmunoPeroxydase Kit, Vector Laboratories). The antigen-antibody complex is developed according to the protocol of the supplier.

Figure 11 shows that a protein having an apparent molecular weight of 21 kDa is secreted by the yeast transformed by pXPHO5. The protein is specifically recognized by the anti-IL-1β antiserum. This protein is not synthesized by the yeast transformed by the control vector pXKN18 (without IL-1β cassette). The secreted protein corresponds to the glycosylated form of IL-1β, which is demonstrated by the reduction of the apparent MW after treatment with the enzyme endo-N-acetylglucosamidase H (Figure 11, lane 3). This host/vector pair *K. waltii*/pXPHO5, which is not yet optimized, secretes approximately 5 mg of IL-1β per liter of culture. The level of secretion of IL-1β by *K. waltii* is higher in the LPi medium than in the HPi medium, which suggests that the activity of the PHO5 promoter is regulated by the phosphate in *K. waltii*, as in *S. cerevisiae*.

6.2 Human serum albumin:

6.2.1 Construction of plasmid pYG140 (Figures 12-14).

A plasmid was constructed which comprises:

- an *E. coli* replicon,
- the gene aph under the control of the promoter k1 of the killer toxin of *K. lactis* (EP361911) in which the HindIII site was eliminated by directed mutagenesis, and
- the gene bla, which confers resistance to ampicillin.
- the gene aph, under the control of the promoter k1, is isolated from plasmid pKan707 (EP361991) in the form of a fragment PstI, which is cloned in the equivalent site of the phage M13mp7. The resulting plasmid is called pYG64 (Figure 12). The HindIII site present in this gene was destroyed by direct mutagenesis according to the method described by Taylor et al. (Nucl. Acid. Res. 13 (1985) 8749). The resulting plasmid is called pYG65. The oligodeoxynucleotide having served for the mutagenesis has the following sequence: 5'-GAAATGCATAAGCTCTTGCCATTCTCACCG-3', and it allows the transformation of the triplet CTT coding for leucine 185 in CTC. To construct plasmid pYG70, the part containing the bacterial replicon of vector pKan707 was isolated by digestion with the enzyme EcoRI and recircularization with T4 DNA ligase to obtain pYG69. The fragment PstI present in the latter vector containing gene aph was then replaced by the mutated equivalent fragment originating from pYG65. The resulting plasmid is called pYG70 (Figure 13).

This plasmid is then digested by EcoRI and relegated in the presence of an EcoRI-NarI-EcoRI adapter containing the following sequence: 5'-AATTCGGCGCCG-3'.

The plasmid obtained is called pYG140 (Figure 14).

6.2.2 Introduction of an expression cassette for albumin (Figure 14)

The gene coding for preproSAH under the control of the promoter and the terminator of the gene PGK of *S. cerevisiae* was isolated in the form of an SalI-SacI fragment from an expression vector pYG19 (EP361991). This fragment was introduced into the corresponding sites of plasmid pYG140 to generate plasmid pYG141.

6.2.3 Construction of expression vector pYG142 (Figure 15)

Plasmids pYG141 and pKW1 are digested by the enzymes NarI and ClaI, respectively. After the ligation, 4 recombinant plasmids are obtained because of the existence of 2 forms, A and B, of pKW1, and of the orientation of the pKW1 part with respect to the pYG141 part.

Figure 15 describes the restriction map of one of these 4 plasmids: pYG142, containing the form B of pKW1.

The other plasmids are called pYG143, pYG144 and pYG145.

One sample of the strain *K. lactis* CBS 6430 was deposited at the CBS in Baarn (Netherlands) pursuant to the conditions of the Treatise of Budapest, on June 4, 1991 under the number CBS 290.91.

Table 1

Vectors	Fragment of pKW1	Shuttle vector	Marker
pBNA1	Bg1II-NheI 2.4Kb	Yip5 *BamHI-NheI	URA3
pNEA2	NheI-EcoRI 2.0kb	Yip5 NheI-EcoRI	URA3
pXXY2	XhoI-XbaI 0.55 kb	Yip5 SalI-NheI	URA3
pXXK3	XhoI-XbaI 0.55 kb	pKan21 SalI-XbaI	Kan^R
pBNB1/A3	BgllII-NheI 1.9 kb	Yip5 BamHI-NheI	URA3
pNEB1	NheI-EcoRI 2.5 kb	Yip5 NheI-EcoRI	URA3

Table 2

Vectors	Linearization site of pKW1	Shuttle vector	Marker
pKWS14	SalI	pKan21*(SalI)	Kan^R
Pkwc11	ClaI	pKan21 (AccI	Kan^R
PKWS 1	SalI	Yip5 (SalI)	URA3

Table 3. Transformation of *Saccharomyces cerevisiae* by vectors derived from pKW1

Vectors	Transformants Ura+ per µg of DNA
pKWS1	4 400
pBNA1	1 200
pBNB1/A3	7 600
pXXY2	4 000
pSK1	4 500

Table 4. Transformation of *Kluyveromyces waltii* by vectors derived from pKW1

Vectors	Replication support	Transformants G418-resistant per µg of DNA	
		$pKW1^+$	$pKW1^-$
pKWC11	pKW1 total	36 00 (98 %)	8 000 (100 %
pXXK3	XbaI-XhoI 540bp de pKW1	35 000 (49 %)	10 000 (2,8 %)
pKWS14	pKW1 total	10 000 (92 %)	8 000 (29 %)

Table 5. Transformation of yeasts of the genus *Kluyveromyces* with the vector pKWC11

Species	Strain	GC%	Frequency of transformation per µg of DNA	Stability of the transformants (%)
K. waltii	CBS 6430	45.6	2400	100
K. thermotolerans	CBS 6340	46.2	4000	25

Claims

1. Plasmid pKW1 isolated from the strain *K. waltii* CBS6430, or any fragment or derivative thereof.

2. Fragment according to Claim 1, characterized in that it is a genetic element.

3. Plasmid characterized in that it comprises all or part of the sequence presented in Figure 3 or a derivative thereof.

4. Cloning and/or expression vector, characterized in that it comprises all or part of plasmid pKW1 of *K. waltii* CBS 6430 represented in Figure 1 or a derivative thereof.

5. Vector according to Claim 4, characterized in that it comprises a genetic element of at least plasmid pKW1.

6. Vector according to Claim 5, characterized in that it comprises the replication origin of plasmid pKW1.

7. Vector according to Claim 4, characterized in that it comprises the totality of plasmid pKW1.

8. Vector according to Claim 4, characterized in that it comprises all or part of the sequence presented in Figure 3 or a derivative thereof.

9. Vector according to Claim 4, 7 or 8, characterized in that plasmid pKW1 is linearized at the level of a functionally neutral restriction site.

10. Vector according to Claim 9, characterized in that it contains restriction site present on plasmid pKW1, or one which was artificially introduced to it.

11. Vector according to Claim 10, characterized in that it contains a restriction site artificially introduced into an intergenic region, and preferably in the region located between the genes B and D, or in the region located between gene D and IR2.

12. Vector according to Claim 10, characterized in that plasmid pKW1 is linearized at the level of the sites ClaI(1), PstI(4608) or EcoRV(3072), the positions being given with reference to Figure 3.

13. Vector according to any one of Claims 4-12, characterized in that it contains, in addition, a heterologous DNA sequence comprising a structural gene which is at least under the control of signals allowing its expression.

14. Vector according to Claim 13, characterized in that the expression signals consist of one or more elements chosen from promoters, terminators, and secretion signals.

15. Vector according to Claim 14, characterized in that the promoters can be regulated.

16. Vector according to Claim 14, characterized in that the promoters are derived from yeast genes, and preferably from glycolytic yeast genes.

17. Vector according to Claim 13, characterized in that the structural gene codes for a polypeptide of pharmaceutical or agro-food interest.

18. Vector according to Claim 13, characterized in that the structural gene codes for a hybrid protein.

19. Vector according to Claim 13, characterized in that the structural gene(s) is (are) genes involved, at the genetic or biochemical level, in the biosynthesis of a metabolite.

20. Vector according to Claim 4, characterized in that it contains in addition an *E. coli* replicon.

21. Vector according to Claim 4, characterized in that it contains in addition at least one selection marker.

22. Recombinant cell containing a vector according to any one of Claims 4-21.

23. Cell according to Claim 22, characterized in that it is a yeast cell.

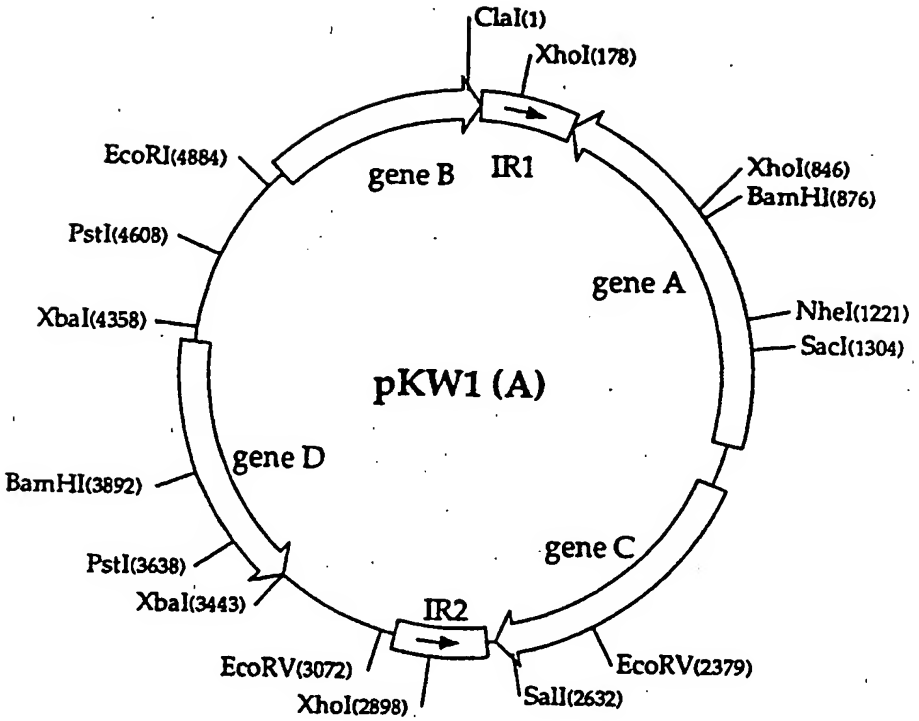
24. Cell according to Claim 23, characterized in that it is a yeast of the genus *Kluyveromyces* or *Saccharomyces*.

25. Method for the preparation of a polypeptide, characterized in that a recombinant cell is cultured according to one of Claims 22-24 and the produced polypeptide is recovered.

26. Method according to Claim 25, characterized in that the polypeptide is chosen from enzymes (such as, notably, superoxide dismutase, catalase, amylases, lipases, amidases, chymosine, etc.), blood derivatives (such as serum albumin, alpha- or beta-globin, factor VIII, factor IX, van Willebrand factor, fibronectin, alpha-1 antitrypsin, etc.), insulin and its variants, lymphokines (such as interleukins, interferons, colony stimulation factors (G-CSF, GM-CSF, M-CSF...), TNF, TRF, etc.), growth factors (such as growth hormone, erythropoietin, FGF, EGF, PDGF, TGF, etc.), apolipoproteins, or antigenic polypeptides for the preparation of vaccines (hepatitis, cytomegalovirus, Epstein-Barr, herpes, etc.).

27. Method according to Claim 26, characterized in that the polypeptide is human albumin or one of its variants or precursors.

28. Use of a recombinant cell according to one of Claims 22-24 as a catalyst in a bioconversion reaction.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EcoRI(4884)
gene B
IR1
XhoI(846)
BamHI(876)
PstI(4608)
gene A
XbaI(4358)
NheI(1221)
SacI(1304)
pKW1 (A)
gene D
BamHI(3892)
gene C
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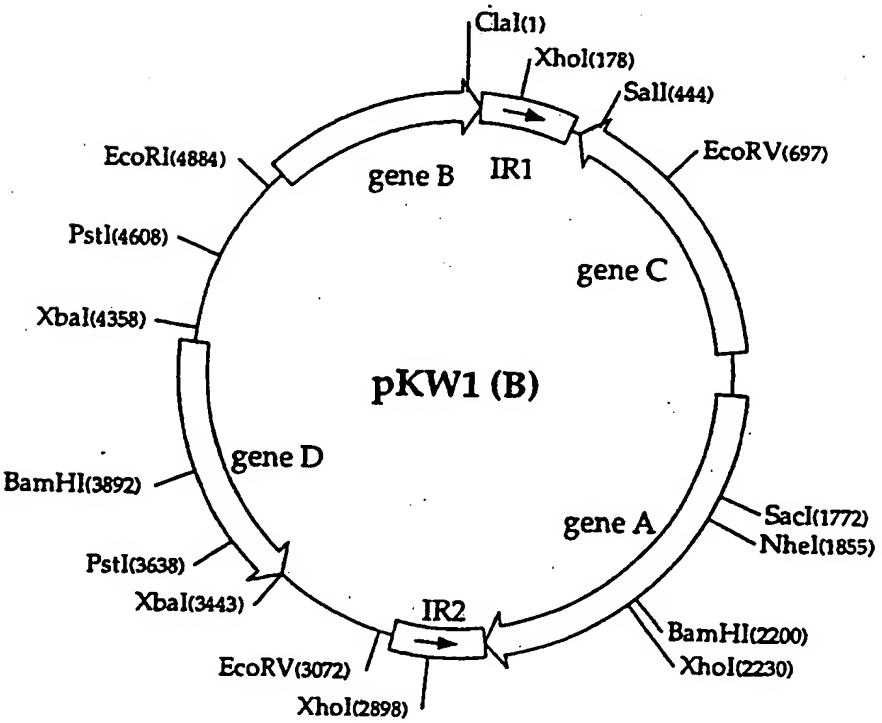
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IR1
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PstI(4608)
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pKW1 (B)
gene D
BamHI(3892)
SacI(1772)
gene A
NheI(1855)
PstI(3638)
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IR2
BamHI(2200)
EcoRV(3072)
XhoI(2230)
XhoI(2898)

FIGURE 1

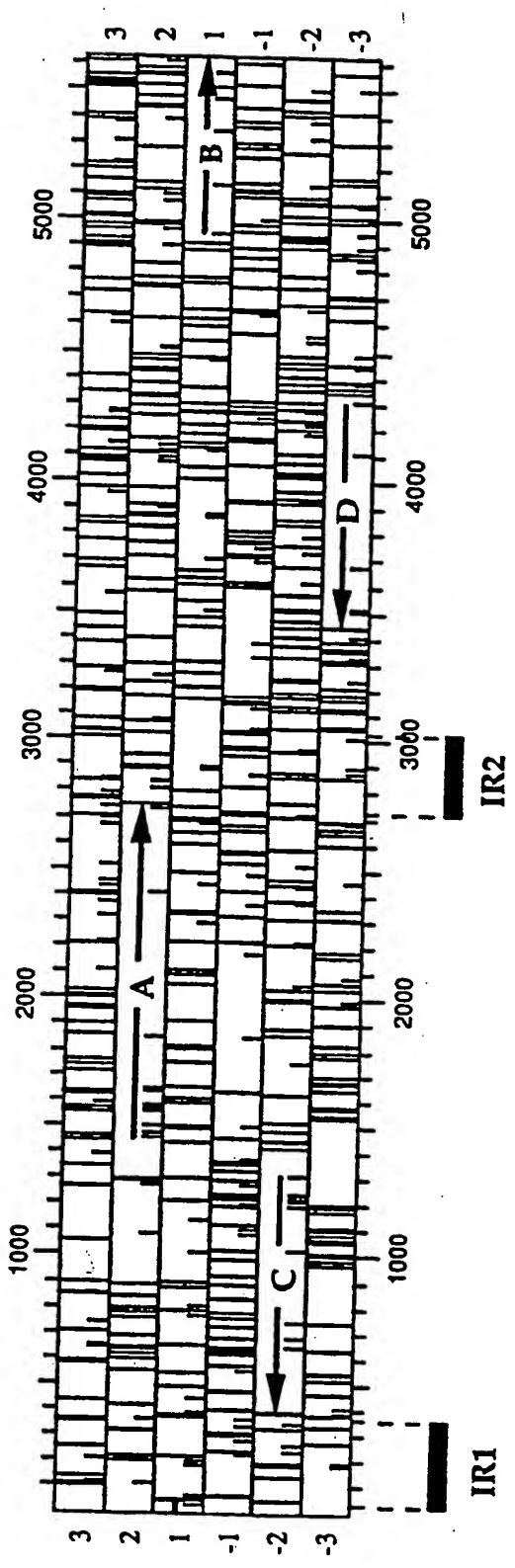

Figure 2

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        |   10      |   20      |   30      |   40      |   50      |   60
   1 ATCGATTGCC AGCAACAGGG TTTTGCGCGT ATGTCTCTTG TGGATACTAT GTAAACAAAA 60
  61 CAATCAATGT ACAAAGAGCA CAGCGGCCGG CAGGTGGGAG GACCCTCTGA TGAGCCGGGG 120
 121 ATATGGCGCT CCCGCGCGTC TAATATCCGG ATTGGACTGG AGGAGGACCA AGGTTTCCTC 180
 181 GAGGTCATGT GCCGCATCAT GAGGGACGAA AAATGGTAAG GAATAGACCA TTCCTTACCA 240
 241 TTTTTCGTCG CCCGTGAGAT TTTCCCATTT CCCGTTCCTT ACCATTTTTC GTTCCGCACG 300
 301 ACCCTATCAC ATTGTATATT GAAATCTACT TCCCTTCATA CCGCTTGGCC ATCGCGTAGG 360
 361 AATGAACATA TGTAGCCCCT CTAGTTCCTC ATGTTGTTCT CAATCGACCC TTCCATCGTG 420
 421 AAGGGATCTC CTCTTGAGGC AATGTCGACG TTCGTACCCT CTTCCTACGG GTCACGGGTT 480
 481 CGTCTGTAAC ACTTCTTCTT GGTCTCCCTC GGCCTCTTTT ACTTGGCTGT GGCCTGGTAT 540
 541 TTTTGAGTTC ATCGAATAGG CGCGGGTTTT CTTTTACTTG AGGCCAGTCT TGAATCTCTG 600
 601 AACCGAGAGA GGTTAGAGAA GTTATTCTTT TTAGATCATT TAACATGTCC TTGATGTCTT 660
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 721 TTACGTTGTG CGCTTCTTCC ATGTTTTCAG GGGCCACATG CTTTAATGCC TGCACTAAAA 780
 781 CACTTCTGAC AAGTGACCAA TCTGCGAATG CAGGCTCGTT CGCCAATATC AATCTCTGTA 840
 841 GAGGAACACC ACGATAGCTT GTTGTGCCCT TGCAGATAGT GTTCAAGAAT AGACTGAGGT 900
 901 AGCCACGTAC GTCGAGCTTT ACAGACCTGG TGCCAACGCC AGAAAGAAGG GCGAAGGGGT 960
 961 CCTCTTCAAT CGGAAAACGG GTCAGGCTAT CCACACGGTA CTCTCTATAT GTACTACGAG 1020
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1081 CCTGCTCATG TGGCTCTTTC TCCCCATTAT CCTCTTCAGA GTTCAGCGAG CCAGATGGCA 1140
1141 GCTCGGCTGG CTCTCGCAGG TACGTGGGAT CGGGCTCACC TCGATCCACC GTCATCTCAT 1200
1201 CAAGATTGCA CATAATCTGC ATCAGTCTCA ACGTCATATC GACCGCGGAA AAGGCGTCCG 1260
1261 AGTAGGAAAC ATGCAGTTTG GAAAGGTCTA ACAGCTTGAG GCAGTGCATC TTGGCCTCTC 1320
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1381 GGATTGCAAA AATGTTGCGA GTACTGCGTG TTCTCGTTCA AAAACAGCGT TCGCTGAAAA 1440
1441 TTTAGGAGTT CAGATGCAAC GCGTAGTGCA AATGGAGGAT TCAAGTTGCA GTAACAATAA 1500
1501 CATGGAACAC CAAGGATCAG TGTTCGAGGA GCTTATCTCC AAAAATCTTA TGAGCCTGAT 1560
1561 GGAAGAATTG ATGTCTATGC TCACTAATGA GAAGGAGTTC CAACGTGAAA GGTTCGCGTC 1620
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1741 AAGATTTGAG TACCATTTGA AGAATCCCAC AGAGCTCAAG GAGACGCTGG ACAAGGCCTT 1800
1801 TAAACCTGTC GTGTTTGAAA TCAAGTCCAA AAAGAAGGTT GTCTCCATGC TGGAGCTAGC 1860
1861 TGCGAAGCTC GACAAAAGGG GATCAGATTC AGCGGGTGGT ACGGTAGCTA GTGAGGTCTC 1920
1921 GAAGCTTGTG CGGGAGGAAG AAATTTGGCT TCTTCTCGTG AACGTGAAGA ATACTATCCA 1980
1981 GGAAAAGGTG CGCAAATCAT CGCTAAGAGC GGAATTGACG TATATTTTGA CAGCCTCATT 2040
2041 CTTCAATTGT TGCAGACATA GCGATCTCAG GAACGCAGAC CCCGCAACAT TTGAGCTGGT 2100
2101 GCCAAATAAG TATGTGGGCC ACGTTGTCCG GGTTTTGGTG TGCGAGACCA AGACCCGAAA 2160
2161 GCCGCGGTTC ATATACTTTT TCCCTGTCAA TACGGCCGCG GATCCTCTAG TAGCGCTTCA 2220
2221 TGATTTGTTC TCGAGCACGT TTCCTTCCAA AAAGAGTCGG ACGTCCGAAA GAAAGCAGGA 2280
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Figure 3 (a)

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2281 ACAGGAATGG CAGATCGTTC GCGACGCATC AATCAACAAC TATGACCGGT TTGTTGGTAA 2340
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2401 CTTGATGAGT TCCTACTTGG CGTATACCCA CCATGGGGAA TGGGTCTCAC CATATGGGAA 2460
2461 TTGGTCAGCT GGGAAAGGAA CCATTGAAAG CAGCGTGGCA AGGGCCAAGT ACGCACATGT 2520
2521 TCAAGCCGAG ATCCCAAGCG ATCTTTTCGC CTTTCTGTCT CAGTACTATC AGGAATCAAA 2580
2581 ATCGGGCGAT TTCGAGCTTA ACGACACCAG CAAAGACCCA ACAAAGCTGG TACGGCACTC 2640
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3001 TGCTCTTTGT ACATTGATTG TTTTGTTTTA GTATTACCTG ACAATCATTT TATATTTTGT 3060
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3961 TTCTTTACAA TAGAAGGTAG CTGCGTATAC TTGTTCCGAA CAACAAAGAT GTGACTCTCT 4020
4021 AGCCGCTTTA CTATTGTGTT CAACACAACG TAGGGTTTTG GCCACTCAAA TGTTAATTCT 4080
4081 GATCGAGGTG CTGATGCTGT GTTCTTGTTG ATCATGTATG AATAGTAGAA CAATGCCAAT 4140
4141 TGAGGATGCT CACTGTATTC CTTAGGCAGT TTGGTGCTGC CATAAGGTGA CACCAGCTCC 4200
4201 TTTAGCTGTG GAACATAGAG TACATCAACA GTTCCTACTA GACAACATTC CTTGAGACAA 4260
4261 TTATGATAGC CATCATGGTC GGTTATCTTA GGTATTTTTT GAGCTTCACT CATCTTCGAA 4320
4321 GCAACCGTGA TAGATTCGAT TGAAGTTCAA ATTCTTATCT AGATGGTGTA TTTGTTTATC 4380
4381 ATAATTTACA ATACAGTCTG TTTTAATTTG CTCGAAGTTG CAGTGAAAGA TGTAAAAAGG 4440
4441 GGCTTCATTT TGCACTACAG ACTTACCCTG ATGTAAAAAA TTTTCATCAT AAAAGCAATT 4500
4501 TCTCGTATAC AAAATGCAGT TTGTTTCGTA TACAGCAAAG CATAGAATAC ACTGTACACT 4560
4561 GCATTTTTAC TACAAGAAAA GTTTTTTTTG CTGCTGTGAC TGGAATGCTG CAGCAGTACC 4620
```

Figure 3 (b)

```
4621 TAGATAGAAA AATGGCATAA AACTGAAATT TTATAGTCAT TTTTCGTGTC TTTCATTCAA 4680
4681 TTTTTTCTCG CAAAAGTTTT CTACAAAAGC AGTCAAAATT GCAATAAGTA TACACTAATT 4740
4741 ACAAGGCGTC TGAGCGCGTG ACTTGAGCGC GTGACGTAAT CGCGAGCTAC GAAAGTTGTT 4800
4801 TGGGCCTCAG ACATCGGATC GACAGAAGAG GTAAGAATAT TGGGAAAGTA CATTCAATTA 4860
4861 CCACAACAAT CGAGAGATTA GTGGAATTCA GTCATTAATG AAAGGTAGGG TAGTCCGCCT 4920
4921 ACTCTTAGTT CTACATTCAG AATACGCATG CAGTCCTCAA GTTCTGATGA AGACGACCTA 4980
4981 ATTGACCCTA TAATTCATCC CAAATCGTTC TATAGGGCGG CTAACGAGAT ACCGAGAGAC 5040
5041 TTTTTGGTTG CGATCCCCAT CAGCGCCTAT GTTTTTAGCG TATTTGCTAA ATCAGTACGA 5100
5101 GATGACTTAC AGGGGCATTT AACGGCGCGA GATATGGCAT TAGCTTATCG TGAACGGCAG 5160
5161 TACTTTCACA GACGCTGGGA GACACGAAAC GACCAGCTTG AGATCCCAGA CTGGTCTGAG 5220
5221 ATCCCAGAAT GGTCTCTCGG GTTACTGGAT CGCCCTCCTT GTATCACTGT GGATCTAGCA 5280
5281 AGAGAACTGC GCGAACTATC TCAAAAATGG ATCGGAGCAT TCGATCTGGG ATCGAAGATG 5340
5341 TCTGGCAGGC TTCTTCTACA GCTTCTGTAC ACCCAGCTGT CATGCCCAAA TGAGGCTGTT 5400
5401 TTCAATAAGC TTTACTGCCT CGTCAAACTA CTAAACAAGG ACGTAAATCG TGCGGACCGA 5460
5461 GCCCTTATGG ACTCTGTATT GAGACCACTT TTTGTCGAGA ATCCATACAT GGGTGAACTA 5520
5521 GATGAAGAAA TACTTGATAA GATATGGTCC AATTTGACTG AAATGAGAAG TCAAGAGTGG 5580
5581 AAACGTATAG CGGAAGCGTT GTCAGGCGAG AATAATGAC                        5619
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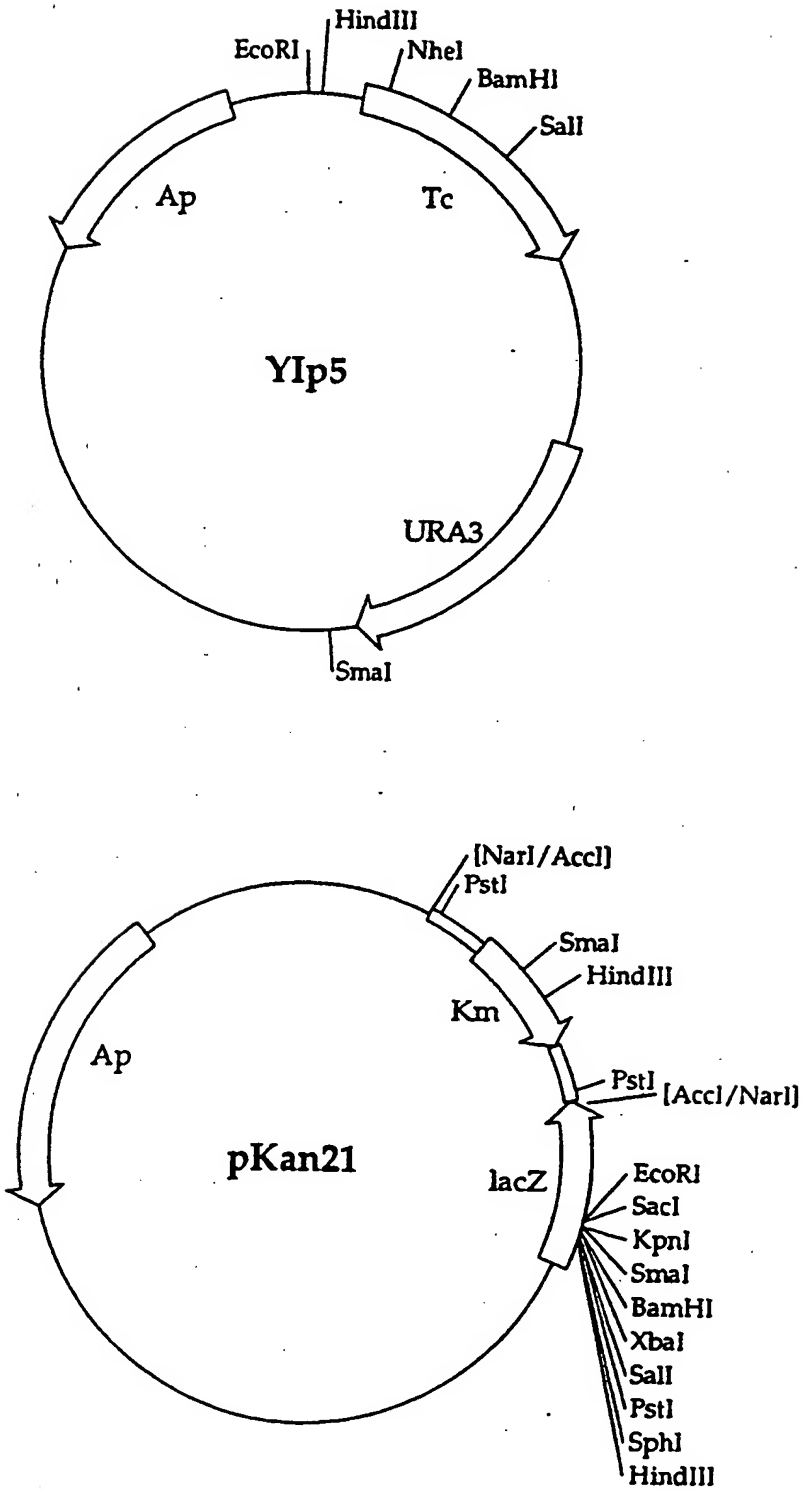
Figure 3 (c)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EcoRI
NheI
BamHI
SalI
Ap
Tc
YIp5
URA3
SmaI
[NarI/AccI]
PstI
SmaI
HindIII
Km
Ap
PstI
[AccI/NarI]
pKan21
lacZ
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII

FIGURE 4

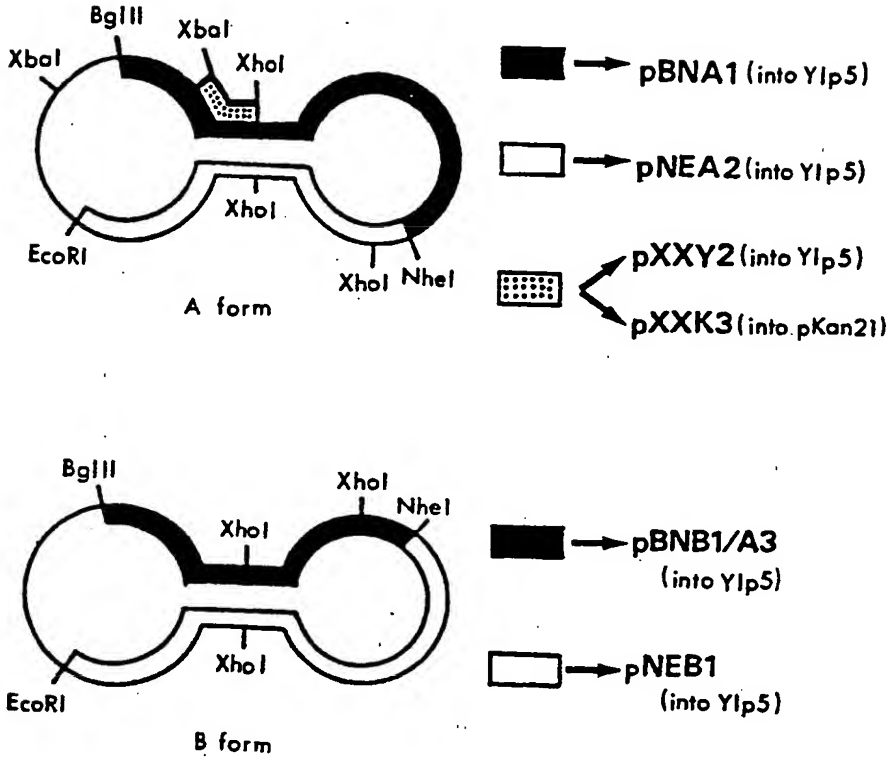

BglII
XbaI
XbaI
XhoI
XhoI
EcoRI
XhoI
NheI
A form
pBNA1 (into YIp5)
pNEA2 (into YIp5)
pXXY2 (into YIp5)
pXXK3 (into pKan21)
BglII
XhoI
NheI
XhoI
XhoI
EcoRI
B form
pBNB1/A3
(into YIp5)
pNEB1
(into YIp5)

FIGURE 5

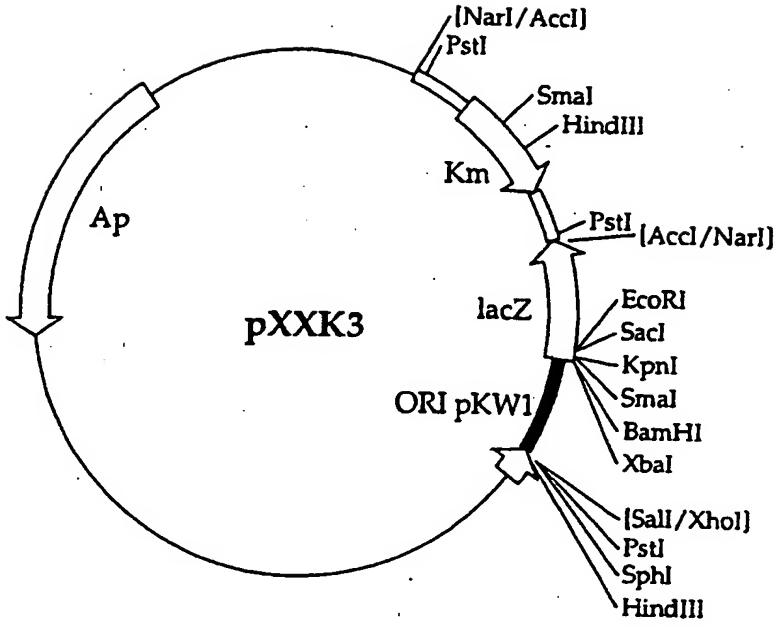
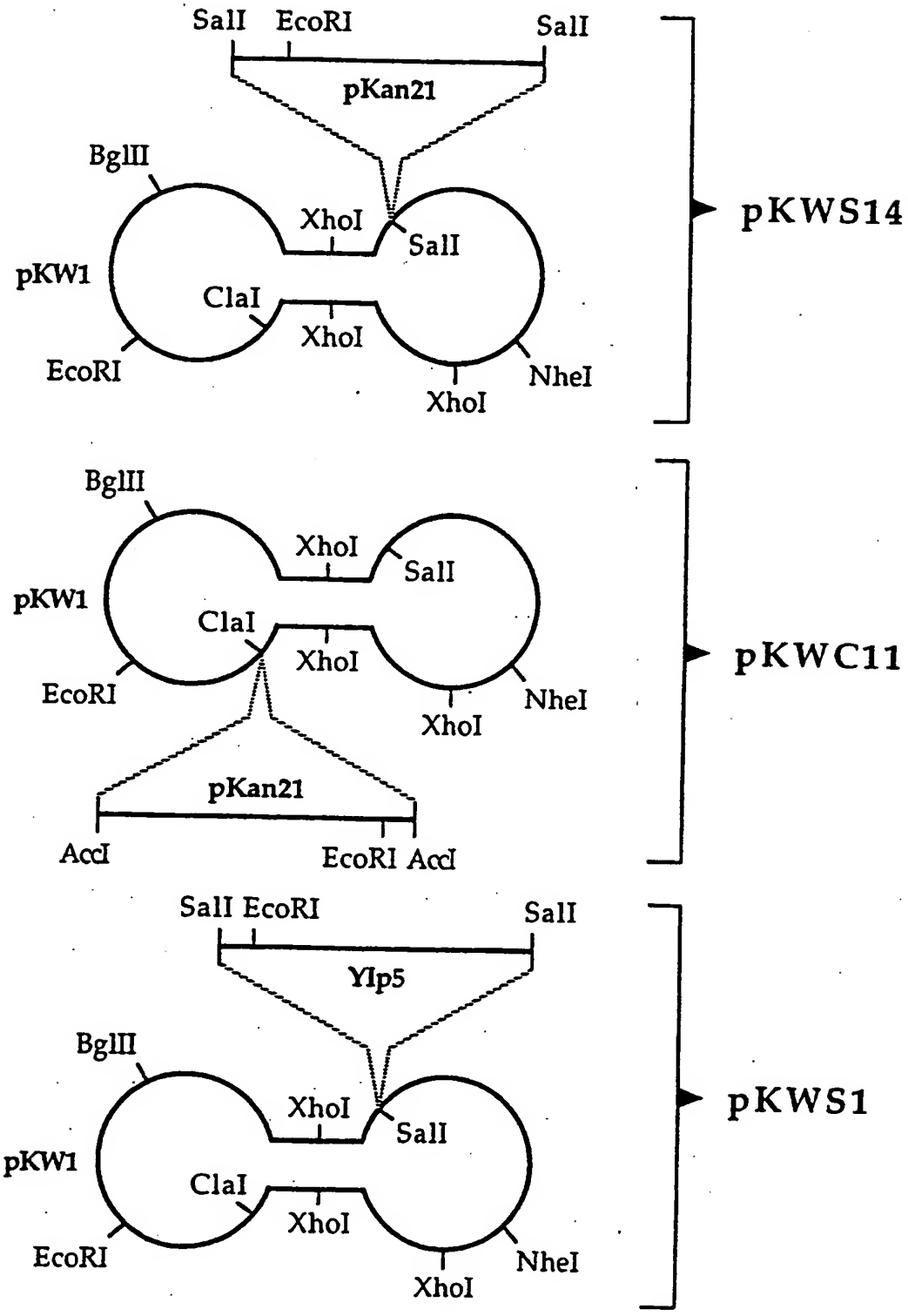

[NarI/AccI]
PstI
SmaI
HindIII
Km
Ap
PstI
[AccI/NarI]
pXXK3
lacZ
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
ORI pKW1
[SalI/XhoI]
PstI
SphI
HindIII

Figure 6


SalI
EcoRI
SalI
pKan21
BglII
XhoI
SalI
pKW1
ClaI
XhoI
EcoRI
NheI
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pKWS14
BglII
XhoI
SalI
pKW1
ClaI
XhoI
EcoRI
NheI
XhoI
pKan21
AccI
EcoRI
AccI
pKWC11
SalI
EcoRI
SalI
YIp5
BglII
XhoI
SalI
pKW1
ClaI
XhoI
EcoRI
NheI
XhoI
pKWS1

Figure 7

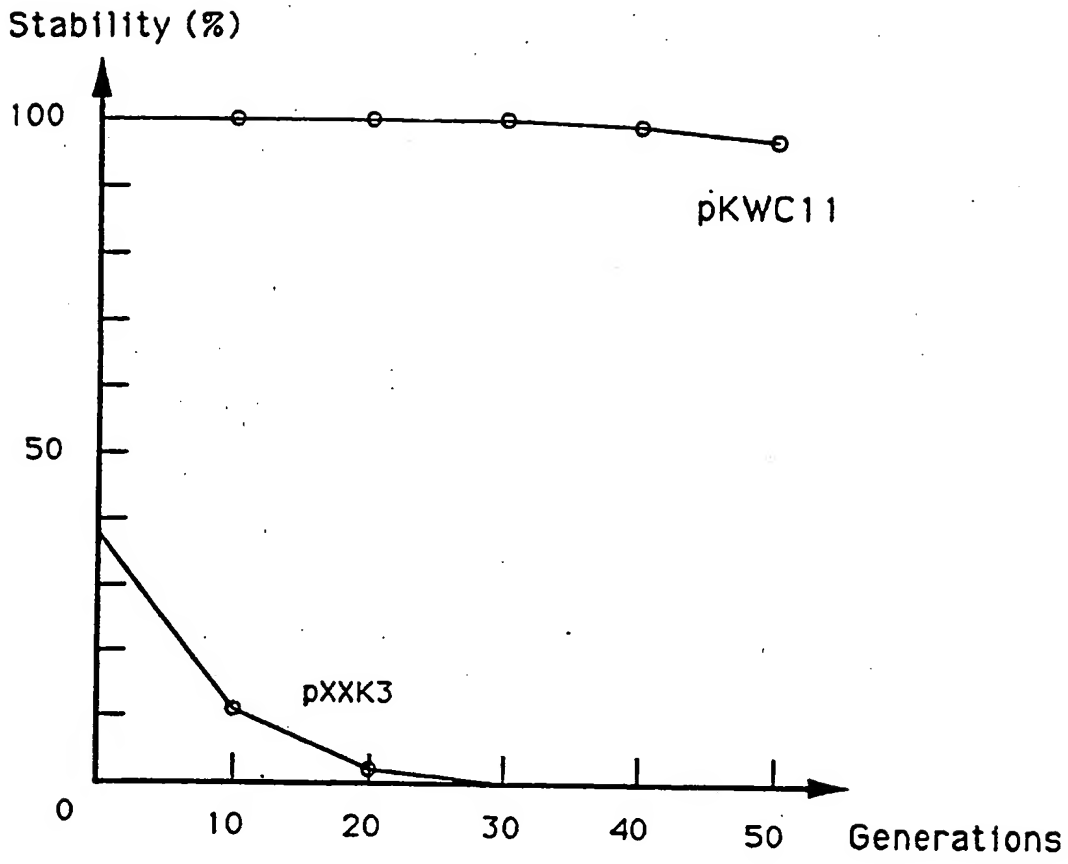


Figure 8

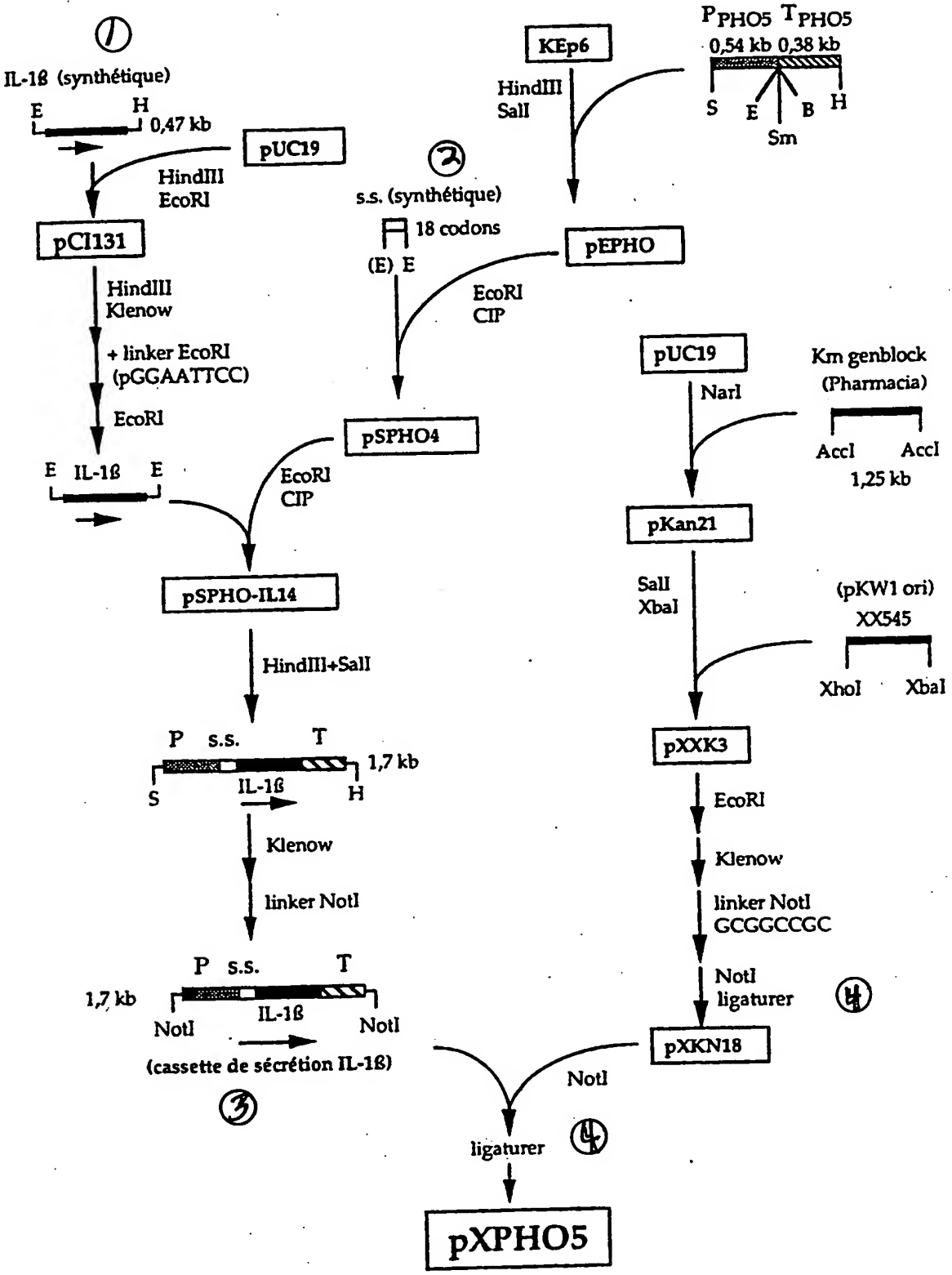
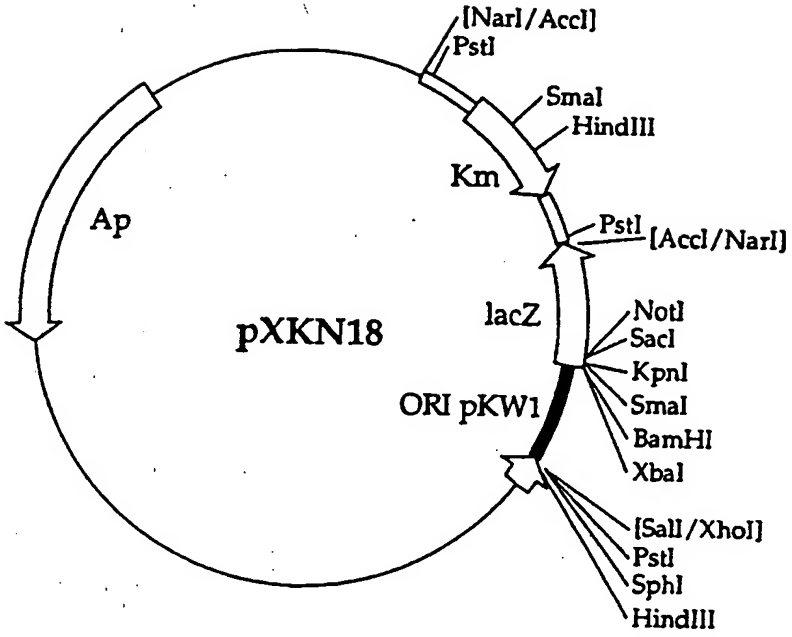


FIGURE 9

Key: 1 IL-1β (synthetic)
2 s.s. (synthetic)
3 (IL-1β secretion cassette)
4 ligate


[NarI/AccI]
PstI
SmaI
HindIII
Km
Ap
PstI
[AccI/NarI]
pXKN18
lacZ
NotI
SacI
KpnI
SmaI
BamHI
XbaI
ORI pKW1
[SalI/XhoI]
PstI
SphI
HindIII

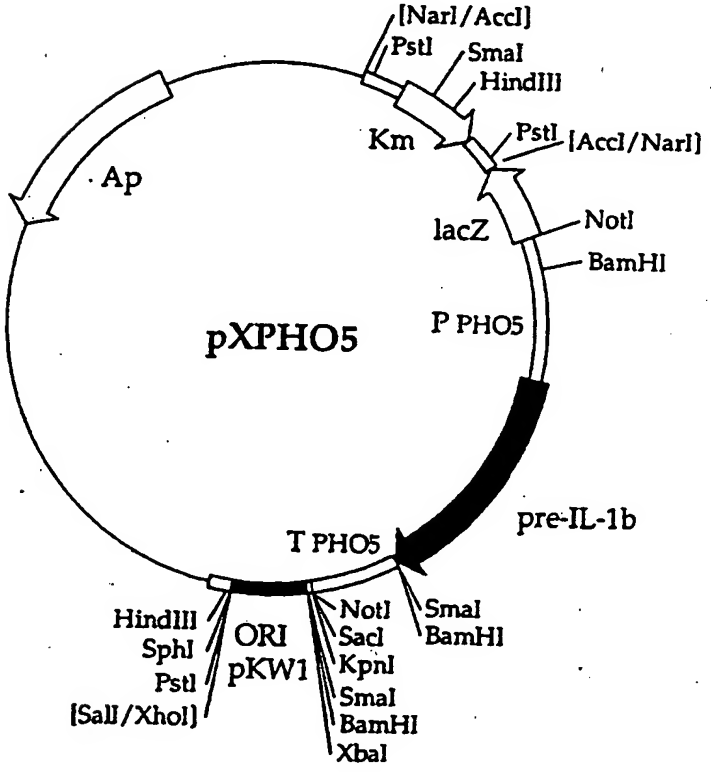
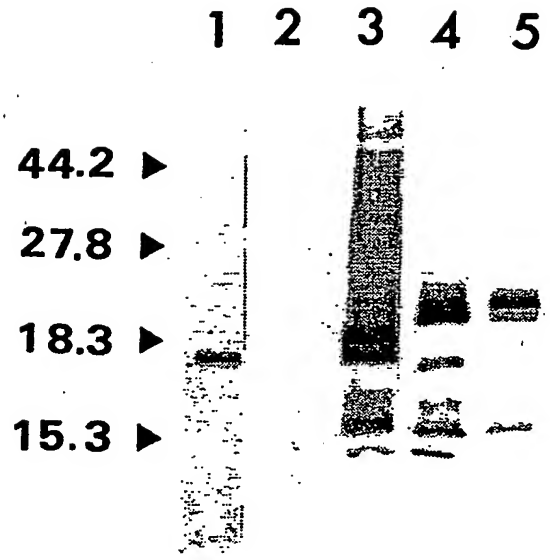

[NarI/AccI]
PstI
SmaI
HindIII
Km
PstI
[AccI/NarI]
Ap
lacZ
NotI
BamHI
pXPHO5
P PHO5
pre-IL-1b
T PHO5
HindIII
SphI
PstI
[SalI/XhoI]
ORI
pKW1
NotI
SacI
KpnI
SmaI
BamHI
XbaI
SmaI
BamHI

FIGURE 10


1 2 3 4 5
44.2
27.8
18.3
15.3

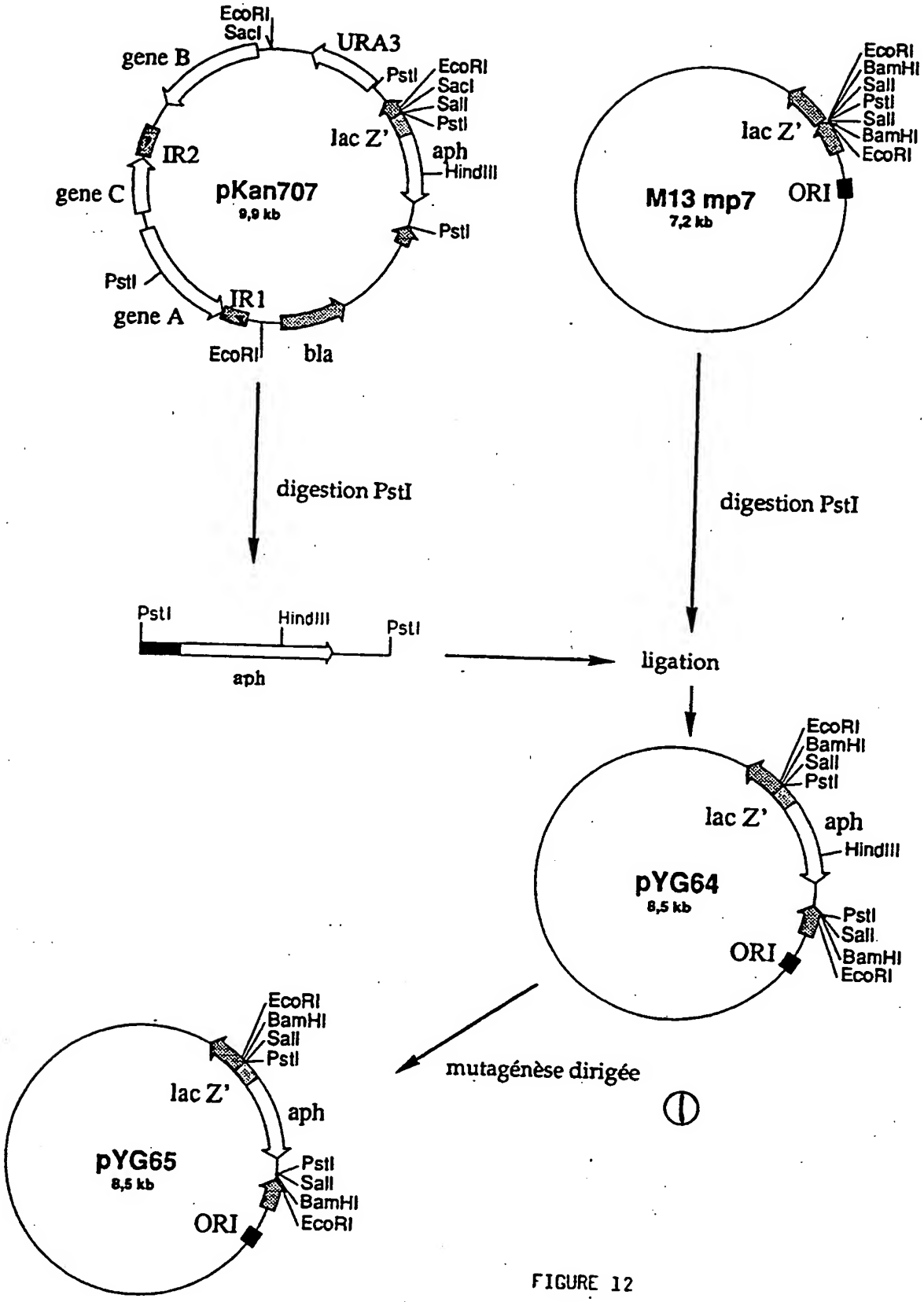


FIGURE 12

Key: 1 Directed mutagenesis

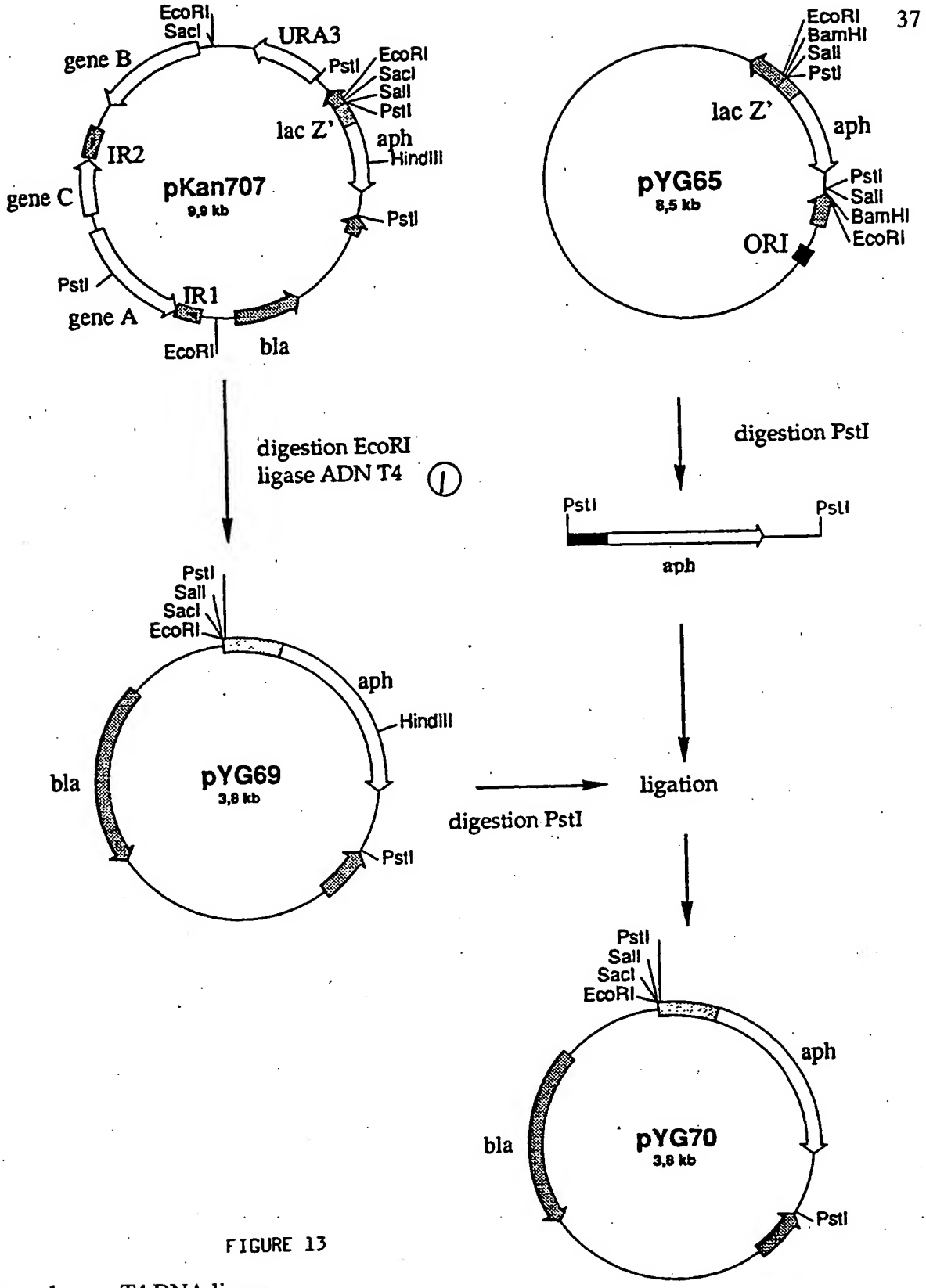
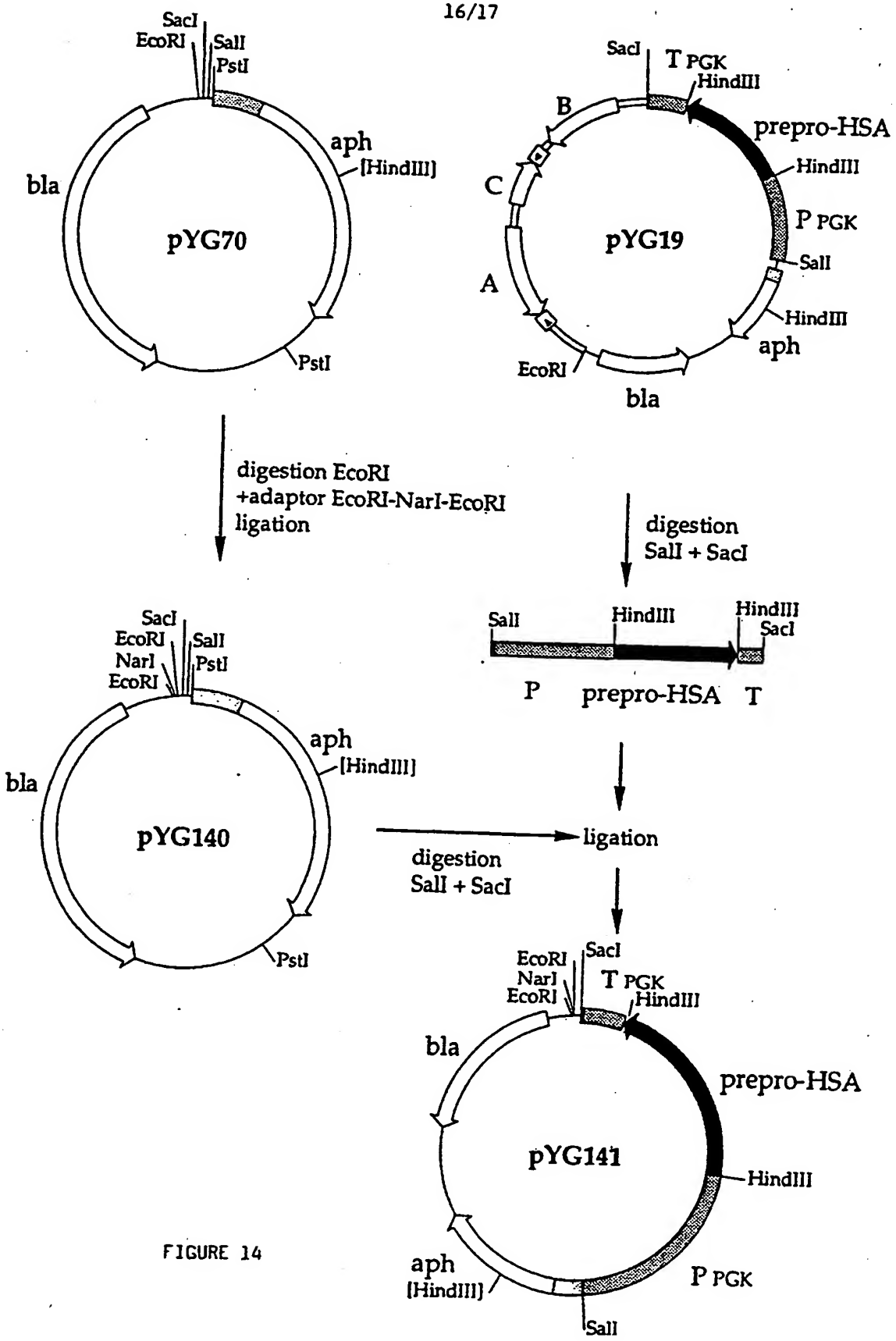


FIGURE 13

Key: 1 T4 DNA ligase


SacI
EcoRI
SalI
PstI
aph
[HindIII]
bla
pYG70
PstI
SacI
T PGK
HindIII
B
prepro-HSA
HindIII
C
P PGK
pYG19
SalI
A
HindIII
aph
EcoRI
bla
digestion EcoRI
+adaptor EcoRI-NarI-EcoRI
ligation
digestion
SalI + SacI
SalI
HindIII
HindIII
SacI
P
prepro-HSA
T
SacI
EcoRI
SalI
NarI
PstI
EcoRI
aph
[HindIII]
bla
pYG140
PstI
ligation
digestion
SalI + SacI
EcoRI
SacI
NarI
T PGK
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bla
prepro-HSA
pYG141
HindIII
aph
[HindIII]
P PGK
SalI

FIGURE 14

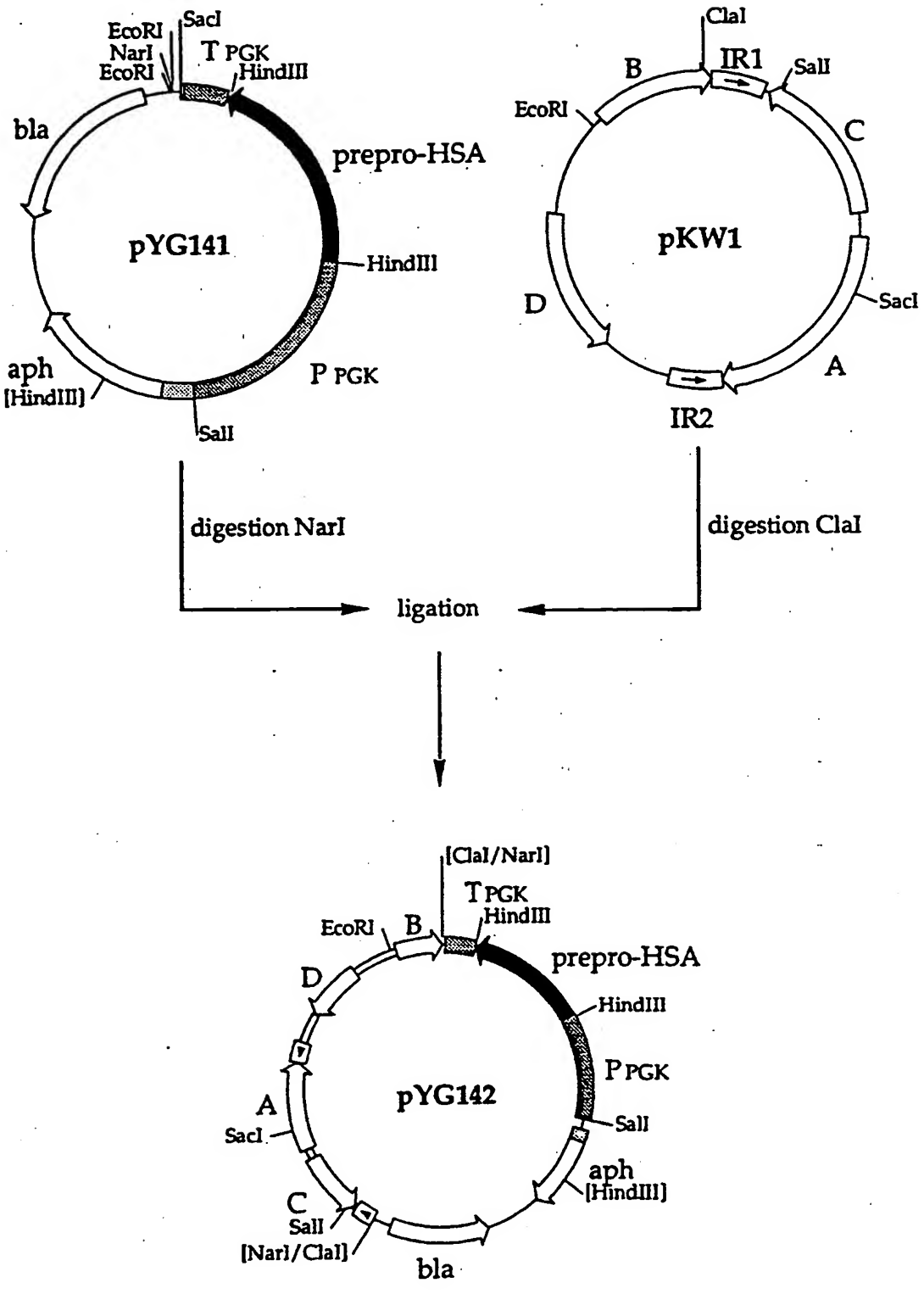
EcoRI
NarI
EcoRI
SacI
T PGK
HindIII
bla
prepro-HSA
pYG141
HindIII
P PGK
aph
[HindIII]
SalI
ClaI
B
IR1
SalI
EcoRI
C
pKW1
D
SacI
A
IR2
digestion NarI
digestion ClaI
ligation
[ClaI/NarI]
T PGK
HindIII
EcoRI
B
prepro-HSA
D
HindIII
P PGK
pYG142
A
SacI
SalI
aph
[HindIII]
C
SalI
[NarI/ClaI]
bla

FIGURE 15

International Application No.: PCT/[illegible]

MICROORGANISMS

Optional sheet pertaining to the microorganism mentioned on page 20, lines 10-11 of the description[1]:

A. Identification of the deposition[2]:

Other depositions are identified on a supplementary sheet[3].

Name of the deposition institute[4]:

Centraalbureau voor Schimmelcultures (CBS)

Address of the deposition institute (including the zip code and the country[4]:

Oosterstraat 1, P. O. Box 273 – 3740 AG Baarn (Netherlands)

Date of deposition[5]:

June 4, 1991

Sequential No.[6]:

CBS 290.91

B. Supplemental indications[7] (to be filled out only if necessary). A separate sheet is attached for the continuation of this information

With regard to the designations under which a European patent is filed, a sample of the microorganism deposited will not be accessible prior to the date of publication of the mention of the grant of the European patent or the date when the application is rejected, withdrawn or considered to be rejected, unless a sample is made available by an expert designated by the requester (rule 28(4) of the EPC).

C. Designated states for which the indications are given[3] (if the indications are not given for all the designated states)

D. Information supplied separately[8] (to be filled out if necessary)

The information listed below will be later subjected to International Office[9] (specify the general nature of the indications, for example, "Sequential No. of the deposit"

E. The present sheet was received with the international application when it was filed (to be verified by the receiving office)

[signature]
N. Saada
(Authorized employee)

Date received (originating from the applicant) by the International Office[11]
(Authorized employee/officer)

Form PCT/RO/134 (January)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR 92/00559

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl.5: C12N15/81

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl.5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FR,A,2 635 115 (RHONE-POULENC SANTE) 9 February 1990	
X,P	JOURNAL OF GENERAL MICROBIOLOGY vol. 138, No.2, February 1992, COLCHESTER pages 337 - 345 X.J. CHEN ET AL. 'Characterization circular plasmid from the yeast Kluyveromyces waltii'	1-28
A	EP,A,0 301 670 (GIST-BROCADES N.V.) 1 February 1989	
	-/--	

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 October 1992 (09.10.92)	26 October 1992 (26.10.92)
Name and mailing address of the ISA/ European Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FR 92/00559

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CURRENT GENETICS vol. 19, No. 3, 1991, BERLIN, GERMANY pages 163 - 167 C. WILSON AND H. FUKUHARA 'Distribution of mitochondrial rl-type introns and the associated open reading frame in the yeast genus Kluyveromyces'	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. FR 9200559 SA 61252

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 09/10/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
FR-A-2635115	09-02-90	AU-B-	623425	14-05-92
		AU-A-	3933289	08-02-90
		EP-A-	0361991	04-04-90
		FR-A-	2649991	25-01-91
		JP-A-	2276589	13-11-90
EP-A-0301670	01-02-89	US-A-	4943529	24-07-90
		AU-A-	2014888	02-03-89
		JP-A-	2000476	05-01-90